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Semi-quantitative evaluation of genotoxic activity of chemical substances and evidence for a biological threshold of genotoxic activity

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Abstract

In Japan, the Chemical Substances Control Law requires evaluation of the genotoxic potential of chemical substances semi-quantitatively by application of a ranking system. During the past 10 years under the law, 1049 new chemical substances were evaluated by a reverse mutation assay in bacteria (RMA) and a chromosome aberration test in cultured mammalian cells (CAT). Of them, 130 (12.4%) were positive in the RMA and 402 (38.3%) were positive in the CAT. Eighty (7.6%) were positive in both tests. Fifty (4.8%) were positive only in the RMA, 322 (30.7%) were positive only in the CAT, and 452 (43.1%) were positive in either the RMA or the CAT. Thus, the tests complement each other in detecting genotoxic substances in vitro. To explore the "threshold" concept, we compared the genotoxic responses of *Salmonella typhimurium* tester strains with and without DNA repair capacity. Recently constructed strains of TA1535 lacking O^6-methylguanine DNA methyltransferase genes (ogt_{ST} or ada_{ST} and ogt_{ST}) showed dose-related increases in the number of revertants induced by *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, methyl methanesulfonate, dimethylnitrosamine, and ethylnitrosourea, while in the same dose ranges the parental strain TA1535 did not. This finding suggests that there is a threshold at which all DNA damage induced by low dose levels of genotoxic chemicals are repaired. That biological threshold seems to exist for both DNA and non-DNA targeting chemicals. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Genotoxic activity; Semi-quantitative evaluation; Biological threshold; Reverse mutation assay; Chromosome aberration test

1. Introduction

The genotoxicity of environmental chemicals is generally evaluated qualitatively rather than quantitatively, that is, it is usually based on positively per se rather than on dose–response relationships or thresh-

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old levels. In Japan, however, the Chemical Substances Control Law [1] provides evaluation of the genotoxic activity of new chemicals semi-quantitatively. The approach is to compare genotoxic activities by ranking parameters of toxicity, such as the number of induced revertants per a given dose (mg/plate) of test substance and the concentration that induces chromosome aberrations in 20% of metaphases examined. The adoption of this approach suggests that Japanese regulatory bodies accept genotoxicity evaluations based on dose–response relationships and thresholds. In this paper, we outline the Chemical Substances Control Law, introduce the methods it proposes, and review the genotoxicity data for over 1000 chemical substances evaluated by that method. Further, we consider on "threshold" as a function of DNA repair capacity, one of the endogenous factors that determine threshold. To avoid semantic argument, we defined here that 'genotoxic' is used only in terms of 'inducing irreversible effects such as gene mutations and/or chromosomal aberrations', 'mutagenic' is only 'inducing gene mutations', and 'clastogenic' is 'inducing chromosomal aberrations'.

2. The Chemical Substances Control law in Japan

The Ministry of International Trade and Industry (MITI) and the Ministry of Health and Welfare (MHW) adopted the Chemical Substances Control Law in 1973 to protect humans from exposure to hazardous substances that have the properties of persistence, bioaccumulation, and chronic toxicological potential via the environment [1,2]. The law, amended in 1986, established a system for evaluating the safety of new chemical substances prior to their manufacture or import. In response to the recent revision of the OECD genotoxicity testing guidelines, the genotoxicity test procedures in the law were revised in 1997, but the law's overall scheme remained as described below.

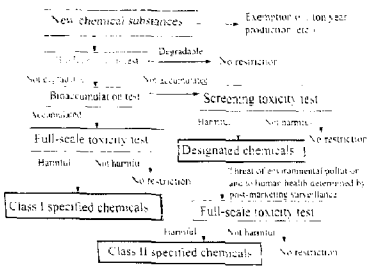

Fig. 1. The Chemical Substances Control Law of Japan.

Table 1
Toxicity tests of the Chemical Substances Control Law

Full-scale toxicity test battery
(1) Chronic toxicity test
(2) Reproductive toxicity test
(3) Teratogenicity test
(4) Mutagenicity test
  (a) Reverse mutation assay in bacteria
  (b) Chromosome aberration test in cultured mammalian cells
  (c) Micronucleus test in rodents
(5) Carcinogenicity test
(6) Toxicokinetic study
(7) Pharmacological study

Screening toxicity test battery
(1) Reverse mutation assay in bacteria
(2) Chromosome aberration test in cultured mammalian cells
(3) 28-Day repeat-dose toxicity test

A chemical substance is considered "new substance" if it is not included in the MITI List of Existing Substances or in the MITI List of Newly Approved Substances. There are some exemptions, such as compounds produced in small quantities (< 1000 kg/year), high molecular-weight substances (polymers), research reagents, medical intermediates, etc. (Fig. 1). As the major objective of the law is the protection of humans from exposure to hazardous substances via the environment, new substances that are readily biodegradable have no restrictions. Substances that are not readily biodegradated are examined for bioaccumulation. Those that accumulate are evaluated by the "full-scale toxicity test" battery, which consists of seven tests (Table 1). Substances found harmful in that battery are classified "class 1 specified chemical substances" and may not be manufactured or imported.

New chemicals that do not degrade but also do not accumulate, are examined by the "screening

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Table 2
Scoring method for 28-day repeat-dose toxicity test

NOEL[a] (mg/kg)	Reversible effect	Irreversible effect[b]			
		1	2	3	≥ 4
≥ 1000	0	1	2	3	≥ 4
≥ 250	1	2	3	4	≥ 5
≥ 25	2	3	4	5	≥ 6
≥ 5	3	4	5	6	≥ 7
< 5	4	5	6	7	≥ 8

[a]NOEL: No observed effect level
[b]1: Slightly irreversible effect, 2–3: moderately irreversible effect, ≥ 4: highly irreversible effect.

toxicity test" battery, which consists of three tests (a reverse mutation assay in bacteria (RMA), a chromosome aberration test in cultured mammalian cells (CAT), and a 28-day repeat-dose toxicity test) for a prediction of chronic toxicity. If chronic toxicity is predicted, the chemical becomes a "designated chemical substances", and the quantity manufactured or imported must be reported to MITI/MHW every year, though labeling is not required. If designated substances are suspected by post-marketing surveillance to be polluting its environment, they are evaluated by a "full-scale toxicity test" battery. Positive substances in the battery are classified as "class II specified chemical substances"; their production and import are limited and special labeling is required.

3. Overall evaluation in the screening toxicity test

Because short-term tests are used to predict chronic toxicity, extrapolation from short-term data must be made. A semi-quantitative system, that is, a ranking/scoring procedure based on toxicity parameters is used for that purpose.

In the 28-day repeat-dose toxicity test, the "no observed effect level (NOEL)" is estimated for each substance based on observed reversible effects. Then, the chemicals are classified into five groups according to the NOEL and are given different scores, as shown in Table 2. Additional scores are added based on irreversible effects. A similar approach is used for genotoxicity tests (Table 3). Positive responses in the RMA are classified into three groups based on the number of induced revertants per a given concentration (mg/plate) of the test chemical. Clastogenic activity in the CAT is expressed as the concentration (mg/ml) at which chromosome aberrations are observed in 20% of metaphases (D_{20}), and positive responses are classified into three groups based on that value. When polyploid induction is observed, the D_{20} is independently calculated, and the evaluation is made in the same way as for clastogenic activity. When both structural and numerical aberrations are induced by a test chemical, the lower D_{20} value is selected for the evaluation. Marginal responses included in the evaluation are also seen in Table 3. The scores in the 28-day repeated dose toxicity test and the genotoxicity tests are used for a preliminary evaluation, independently or supplementally, on a case-by-case basis.

Overall evaluation of test chemicals by the MHW is made using all available data, such as outcomes in the 28-day repeat-dose toxicity test and the genotoxicity tests, information on chemical structure, and biodegradation and bioaccumulation data. Informa-

Table 3
Scoring evaluation in mutagenicity tests

Chromosome aberration test (D_{20}^{a})	Reverse mutation assay (MA[b])				
	−	±	− ($< 10^2$)	+ ($\geq 10^2$)	− ($\geq 10^3$)
−	0	1	2	3	5
±	1	2	3	4	6
+ (≥ 1)	2	3	4	5	7
− ($\geq 10^{-2}$)	3	4	5	6	8
+ ($< 10^{-2}$)	5	6	7	8	10

[a]D_{20}: The estimated dose (mg/ml) in which aberrations are observable in 20% of metaphases.
[b]MA: Mutagenic activity (revertants/mg) indicated as the number of induced revertants per a given dose (mg/plate).

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tion on manufactured or imported quantities, exposure route, etc., are also considered.

From October 1988 to August 1998, 1049 new chemical substances were evaluated by the screening toxicity test battery, and 385 (36.7%) were classified as designated chemical substances.

4. Comparison of mutagenic and clastogenic potential of 1049 new chemicals

The majority of the RMAs were performed with the standard procedure recommended by the Chemical Substances Control Law using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 *uvrA*. A few tests were carried out with TA1538 additionally or without WP2 *uvrA*. Exceptionally, new strains such as TA97, TA102, or WP2 *uvrA* (pKM101) were used. The majority of the CATs were performed with a Chinese hamster cell line (CHL/IU) according to the standard procedure, but some tests were carried out with the previous OECD procedures (only short treatments) using other Chinese hamster cell lines (CHO or V79) or cultured human lymphocytes.

Of the 1049 new chemicals tested, 130 (12.4%) were positive in the RMA, 402 (38.3%) were positive in the CAT, and 80 (7.6%) were positive in both tests. Fifty (4.8%) were positive only in the RMA, while 322 (30.7%) were positive only in the CAT. Overall, 452 (43.1%) were positive in the RMA and/or the CAT. Thus, the tests were complementary in detecting genotoxic substances in vitro.

Of the 130 RMA-positive chemicals, 25 (19.2%) were strongly mutagenic, that is, the mutagenic activity was ≥1000, and 24 (96%) of those were classified as designated chemical substances (Fig. 2). One chemical was not classified as a designated chemical substance in spite of its high mutagenic activity because the induced response was not dose-related. Thirty-eight (29.2%) chemicals showed moderate mutagenic activity (1000 > MA ≥ 100), and 33 (87%) of those were called designated chemical substances. Fifty-six (43.1%) had relatively weak mutagenic activities (< 100), and 37 (66%) of those were classified as designated chemical substances. Marginal responses (less than twice the number of revertants as the negative control) were observed for 11 (8.5%) chemicals, and seven (64%) of those were designated chemical substances. Finally, out of the 130 RMA-positive chemicals, 101 (77.7%) were designated chemical substances. In the 919 RMA-negative chemicals, 284 (30.9%) were designated chemical substances. The above-findings indicate that quantitative evaluation of mutagenicity contributes significantly in the judgment of designated chemical substances, though the designated chemical substances with negative or marginal RMA responses

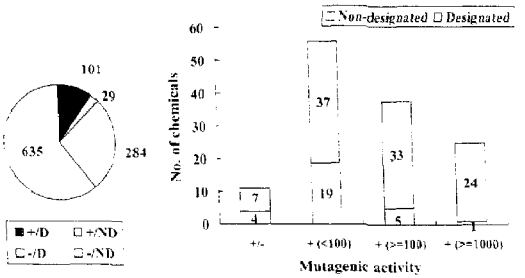

Fig. 2. Results of 1049 new chemicals evaluated by the bacteria reverse mutation assay. +: Positive results, −: negative results, ±: marginal responses. Mutagenic activity: < 100: less than 100; ≥ 100: 100 or more but less than 1000; ≥ 1000: 1000 or more. D: Designated chemicals, ND: non-designated chemicals.

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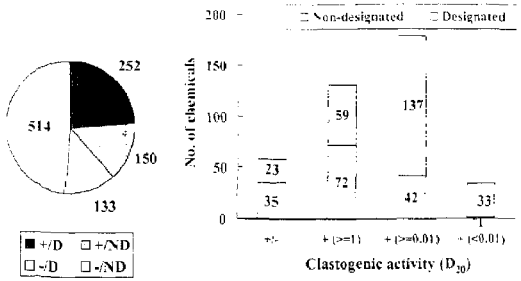

Fig. 3. Results of 1049 new chemicals evaluated by the chromosome aberration test. +: Positive results, −: negative results, ±: marginal responses. D_{20}: ≥ 1: 1 or more, ≥ 0.01: 0.01 or more but less than 1, > 0.01: less than 0.01. D: Designated chemicals, ND: non-designated chemicals.

are, of course, judged mainly on the CAT and/or the 28-day repeat-dose toxicity test.

Of the 402 CAT-positive chemicals, 34 (8.5%) showed strong clastogenic activities ($D_{20} < 0.01$), and 33 (97%) of those were classified as designated chemical substances (Fig. 3). One-hundred and seventy-nine (44.5%) chemicals showed medium clastogenic activities ($0.01 \leq D_{20} < 1.0$), and 137 (77%) of those were called as designated chemical substances. One-hundred and thirty-one (32.6%) had weak clastogenic activities ($1 \leq D_{20}$), and 59 (45%) of those were designated chemical substances. Marginal responses (chromosome aberrations were observed in less than 10% of metaphases) were found for 58 (14.4%) chemicals, and 23 (40%) of those were designated chemical substances. Finally, out of the 402 CAT-positive chemicals, 252 (62.7%) were designated chemical substances. Out of the 647 CAT-negative chemicals, 133 (20.6%) were designated chemical substances.

5. Evidence for a biological threshold based on DNA repair capacity

The organizer of the ECETOC-EEMS Symposium, "Threshold-Mediated Mechanisms in Mutagenesis" on September 7, 1998, in Salzburg, Austria, defined biological threshold as "a concentration of a chemical which although present at the target in finite amounts, does not produce any damage through its inability to perform at or below this concentration, the necessary biochemical reactions. In other

Table 4
Mutagenicity of various alkylating agents in strains possessing different repair system of alkylation damage
The mutagenicity test was performed with 5–9 doses. Chemicals were assayed with four strains in parallel. The number of induced His^+ revertants per nmol or μmol was calculated at every dose where the actual numbers of His^+ revertants per plate were more than twice as many as those spontaneous His^+ revertants per plate; the highest value for each chemical and strain is indicated. The numbers in parentheses represent the relative values when the number of His^+-induced revertants per nmol or μmol of TA1535 is assigned as 1.0. In case the numbers of His^+ revertants per plate at any dose did not exceed twice the numbers of spontaneous revertants, ND is shown in place of the values.

Chemicals	Induced His^+ revertants per nmol or μmol			
	TA1535	YG7100	YG7104	YG7108
	Wild type	Δada_{ST}	Δogt_{ST}	Δada_{ST} Δogt_{ST}
MNNG	61 (1.0)	146 (2.4)	717 (12)	3839 (63)
ENNG	0.1 (1.0)		953 (9530)	1030 (10,300)
PNNG	87 (1.0)		4704 (54)	3808 (44)
BNNG	62 (1.0)		1504 (24)	1319 (21)
MNU	2 (1.0)	5 (2.5)	17 (8.5)	34 (17)
ENU[a]	321 (1.0)		9017 (28)	9227 (29)
MMS[a]	ND	ND	936	7619
EMS[a]	ND	ND	9439	11,178

[a] The number of His^+ revertants per μmol instead of nmol is indicated.

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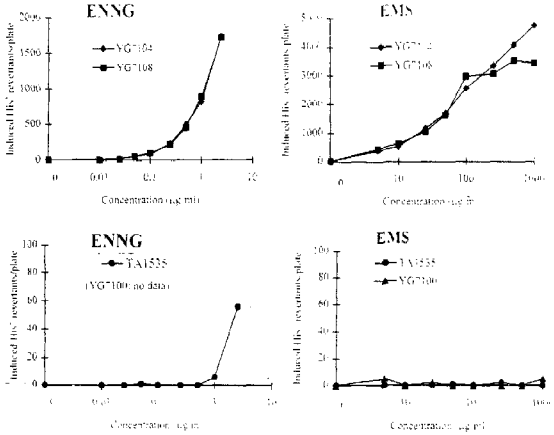

Fig. 4. Mutagenic responses of *S. typhimurium* TA1535 strains with and without O^6-methylguanine DNA methyltransferases to ENNG and EMS. : ●TA1535, : ▲YG7100, : ◆YG7104, : ■YG7108. (For ENNG, no data for YG7100 was available.)

words, the chemical is present, and can interact with the target, but no adverse consequence is induced."

As almost all normal organisms have pre-determined DNA repair capacity, such capacity is con-

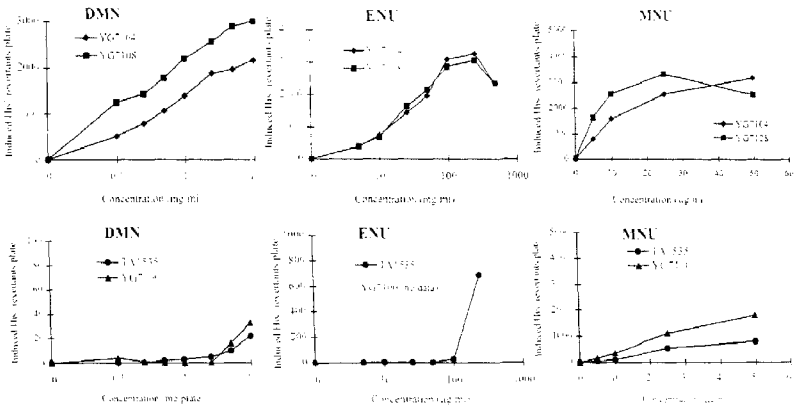

Fig. 5. Mutagenic responses of *S. typhimurium* TA1535 strains with and without O^6-methylguanine DNA methyltransferases to DMN, ENU and MNU. : ●TA1535, : ▲YG7100, : ◆YG7104, : ■YG7108. (For ENU, no data for YG7100 was available.)

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ceivably one of the endogenous mechanisms which might mediate a biological threshold. We compared the mutagenic responses to alkylating agents of *S. typhimurium* strains with and without the ability to remove alkyl groups from O^6-methylguanine and O^4-methylthymine. We used *S. typhimurium* new strains that lack the O^6-methylguanine-DNA methyltransferase genes: YG7100 (TA1535 lacking ada_{ST}), YG7104 (TA1535 lacking ogt_{ST}), and YG7108 (TA1535 lacking ada_{ST} and ogt_{ST}) [3].

Mutagenic potential to various alkylating agents in strains possessing different repair system of alkylation damage is shown in Table 4. *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) increased His^+ revertants at low concentration (0.03 μg/plate) in YG7104 and YD7108, while in TA1535 did at about 100-fold high concentration (2.5 μg/plate) (data not available for YG7100) (Fig. 4). Ethyl methanesulfonate (EMS) induced dose-related increases of His^+ revertants in YG7104 and YG7108, but not in TA1535 and YG7100 (Fig. 4). Dimethylnitrosamine (DMN) and ethylnitrosourea (ENU) yielded similar dose–responses in those four strains (YG7100 data not available for ENU) (Fig. 5). Methylnitrosourea (MNU) showed steep dose–responses in YG7104 and YG7108, but relatively weak responses in YG7100 and TA1535 (Fig. 5). We postulate that if a lower concentration of MNU is used in TA1535, a no-response dose-range will be detected. That YG7100 and TA1535 showed similar responses indicates that Ogt_{ST}, not Ada_{ST}, plays a key role in repairing the alkylation damage induced by these chemicals. The finding that TA1535 has a non-response range, in contrast to strains lacking DNA repair capacity, suggests that the strains with normal DNA repair capacity have a biological threshold to alkylating agents that act directly DNA.

6. Discussion

Recently, Broschinski et al. [4] summarized the genotoxicity test results for 776 new chemicals introduced in Germany between 1982 and 1997. Nearly all substances were evaluated by the RMA and 13.4% were positive. Of the 133 chemicals tested in the CAT, 25.2% were positive. More than 70.8% of the RMA-positive chemicals were negative in the CAT, and more than 83.3% of the CAT-positive chemicals were negative in the RMA. In Japan during the last decade, 1049 new chemical substances were evaluated by both tests; 12.4% were positive in the RMA and 38.3% were positive in the CAT. 38.5% of the RMA-positive chemicals were negative in the CAT and 80% of the CAT-positive chemicals were negative in the RMA. The proportions of mutagenic chemicals, clastogenic chemicals, and non-mutagenic-clastogenic chemicals were similar in the two countries, but the proportion of non-clastogenic–mutagenic chemicals was much higher in Germany (70.8%) than in Japan (38.5%). We agree, however, with the conclusion of Broschinski et al. [4], "With respect to a sensitive detection of genotoxic potentials of substances, the combination of the bacterial gene mutation test and in vitro chromosomal aberration test is appropriate for basic testing."

In the chemical substances control law of Japan, the parameters for mutagenic and clastogenic potential used are directly related to the concentrations at which gene mutations or chromosome aberrations are induced. When gene mutations or chromosome aberrations are induced at low concentrations, resulting in a high mutagenic activity or low $D_{2.}$, the test chemical is regarded as potentially hazardous to humans. On the other hand, positive responses at high concentrations will frequently result in negligible activity levels because such a situation will normally not be the case in in vivo, as is frequently supported by the high NOEL obtained from 28-day repeat-dose toxicity tests. In such cases, the threshold concept seems to be practically accepted.

At present, the argument for a threshold is generally made for non-DNA-targeting chemicals that affect enzymes relating to DNA synthesis, such as topo isomerase II or DNA polymerases, and proteins relating to mechanical apparatuses of cell division, such as spindles and centrioles [5]. DNA precursor analogues and inhibitors of specific cell cycle stages also may induce DNA damage through the disruption of normal DNA synthesis. Functional disruption by non-DNA-targeting chemicals may be non-linear in effect.

DNA-targeting chemicals are believed to induce effects in a linear, dose-dependent fashion with no threshold. However, most DNA damages is thought

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to be repaired, and irreversible effects, such as point mutations and chromosome aberrations, will be fixed from a minor fraction of unrepaired or misrepaired DNA damage. The differing mutagenic responses of TA1535 strains with and without O^6-methylguanine DNA methyltransferases suggested that the strains having normal DNA repair capacity may have a threshold for mutation induction. At some concentrations, the strains deficient in DNA repair capacity showed clear dose-related mutagenic responses. The same concentrations, however, did not induce mutations in the DNA repair-proficient strains. These findings suggest that there may be a threshold below which all induced DNA damage is repaired. The above-consideration encourages us to consider that the threshold will be available for both DNA and non-DNA targeting chemicals.

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